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Evidence for Intercellular Coupling and Connexin-like Protein in the Luminescent Endoderm of *Renilla koellikeri* (Cnidaria, Anthozoa)

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Abstract. Gap junction plaques are abundant in Hydrozoa, where they play an important role in signal propagation through epithelia and nerve nets, but they have not been found in the two other classes of Cnidaria, the Scyphozoa and the Anthozoa. Here several lines of evidence are presented that point to the existence of intercellular coupling in tissues of the anthozoan *Renilla koellikeri*, especially in the luminescent endoderm. Dye-exchange experiments show that calcein vital stains spread between cultured cells after their reassociation. Polyp luminescence evoked by KCl depolarization, electrical stimulation, or β-adrenergic agonists was largely and reversibly suppressed in the presence of the gap junction uncouplers octanol, heptanol, and low pH sodium acetate. A connexin43-like protein was isolated on Western blots of *R. koellikeri* membrane extracts by using a monoclonal connexin-43 antibody. Loading this antibody in *R. koellikeri* tissues resulted in the suppression of luminescence evoked by electrical stimulation. Immunohistochemical investigations using this antibody revealed mostly punctate immunostaining associated with endodermal cells of the luminescent tissue and with the mesogleal nerve net. Electron microscopic observations confirmed the absence of conventional gap junction plaques in these tissues, but revealed the presence of tiny zones of close membrane apposition between light-emitting and other endodermal cells, with gaps of 2–4 nm. Taken together, these results are consistent with the notion of the existence in *R. koellikeri* of intercellular coupling (1) involved in local transmission of luminescence signals, and (2) mediated by connexin43-based connexons that are not assembled into typical gap junction plaques.

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Introduction

Gap junction channels are recognized as important pathways for intercellular communication subserving electrical and metabolic coupling in metazoan tissues (Paul, 1995). The wide range of roles attributed to gap junctions—maintenance of homeostasis, mediation of hormonal responses, and coordination of cellular activities in epithelial and neural conduction as well as in development—is reflected in the wide distribution of these channels among metazoan phyla (Revel, 1988). Within the phylum Cnidaria, however, gap junctions were reported to be abundant in the Hydrozoa, but absent in the Scyphozoa and the Anthozoa (Mackie *et al.*, 1984). Because cnidarians appear to be the lowest metazoans to possess gap junctions and nervous systems, this sharp dichotomy raises questions about the scope of intercellular communication in the classes Scyphozoa and Anthozoa.

In the Anthozoa, effector activities appear to be coordinated solely by simple nerve nets utilizing chemical synapses (Josephson, 1974; Satterlie and Spencer, 1987). One of these activities is bioluminescence in the colonial pennatulid *Renilla koellikeri* (Parker, 1920; Nicol, 1955; Anderson and Case, 1975), a behavior that probably serves as a deterrent to predators (Morin, 1983). Luminescence spreads over the colony as a wave that mirrors the spatio-temporal dynamics of excitation in the underlying colonial nerve net (Nicol, 1955; Anderson and Case, 1975). What is poorly understood, however, is how nerve-net activity translates into local luminescence ex-

citation. A putative transmitter, epinephrine (E), specifically elicits luminescence in *R. koellikeri* (Anctil *et al.*, 1982) and has been detected in this species both by radioenzymatic methods and by high-performance liquid chromatography (HPLC) (De Waele *et al.*, 1987; Pani and Anctil, 1994a). Although the cellular distribution of E is still unknown, the presence of norepinephrine (NE)-immunoreactive nerve-net neurons (Pani *et al.*, 1995) and *in vivo* as well as *in vitro* evidence of enzymatic conversion of NE to E (Pani and Anctil, 1994b) support the existence of E-containing nerve-net neurons.

Several lines of evidence suggest that the adrenergic mediation of luminescence is indirect. First, E is ineffective in eliciting light emission directly from dissociated photocytes, the light-emitting endodermal cells (Germain and Anctil, 1988). Second, the characterized β2-like adrenoceptors that have been associated with luminescence (Awad and Anctil, 1993) were localized by both autoradiography and *in situ* hybridization on "granular" cells adjacent to photocytes, but not on the photocytes themselves (Awad and Anctil, 1994). Lastly, although the endoderm of the muscular mesenteries of *R. koellikeri* is well innervated (Anderson, 1976), innervation of the circular musculature and of the luminescent endoderm is sparse (Satterlie *et al.*, 1976; Germain and Anctil, 1988). This raises the possibility that local transmission of nerve-net signals within the luminescent endoderm may involve pathways other than neural, possibly cell-to-cell coupling.

In another cnidarian luminescent effector where control appears to be indirect, that of the hydrozoan colony *Obelia*, photocytes depend on calcium entry through gap junctions from neighboring cells to trigger light emission (Dunlap *et al.*, 1987; Brehm *et al.*, 1989). Consideration of this alternative model of indirect control prompted us to examine the role of gap-junction-like coupling in luminescence regulation of the *Renilla* system and, more generally, to investigate the presence of specialized junctions and connexin-like proteins in the tissues of *R. koellikeri*. Having used multiple approaches to address this issue, we now report evidence that intercellular coupling involving a protein of the connexin family exists in *R. koellikeri*, and that this coupling plays a role in signal transmission throughout the luminescent endoderm.

Materials and Methods

Colonies of the sea pansy *Renilla koellikeri*, comprising polyps (autozooids and siphonozooids) rooted in a colonial tissue mass (rachis) prolonged by a peduncle, were obtained from Marinus Inc. (Long Beach, CA) and maintained in recirculated and aerated filtered seawater (Instant Ocean) at pH 7.6–8.0 and 13–15°C. The unfed animals were exposed to a photoperiodic cycle of 12 h: 12 h and used within 10 days of their arrival.

Effects of uncoupling agents on luminescence

Rachis pieces of about 5 × 5 mm (including autozooid polyps) were excised from colonies anesthetized in a 1:1 mixture of 0.37 *M* $MgCl_2$ and seawater. The excised tissues were maintained in artificial seawater (ASW: NaCl 395 m*M*, KCl 10 m*M*, $CaCl_2$ 10 m*M*, $MgCl_2$ 50 m*M*, and HEPES 10 m*M*, pH 8) until used. Luminescent responses were recorded by placing rachis pieces in a vial containing 1 ml of ASW, inserting the vial in the well of an LKB 1250 luminometer, and measuring the light output after injection of 0.5 ml of 0.53 *M* KCl in the vial with an LKB 1290 automatic dispenser. In some experiments, the β-adrenergic agonists isoproterenol and atenolol (both at 10 μM) were substituted to the KCl solution. The luminescence signal (in millivolts) was digitized and stored for analysis with a BIOPAC MP100WS data acquisition system (Goleta, CA). Luminescent responses were quantified by integrating areas under the succession of flash curves recorded over a fixed period of time.

Three agents known to block gap junctions were used: 500 μM heptanol or 250 μM octanol in ASW, and sodium acetate seawater at pH 6.5 (Johnston *et al.*, 1980; Turin and Warner, 1980; Spray *et al.*, 1982). After one KCl-induced response was obtained in regular ASW, the polyps were transferred to any of the above solutions for 10 min and their response to KCl was again tested. The polyps were washed three times, 1 min per wash, in fresh ASW and subjected to another KCl challenge (recovery). The effect of these uncouplers on electrically induced luminescence was also tested. The same protocol was used except that trains of 10-ms pulses at 2 Hz delivered through a silver wire electrode in the luminometer vial were substituted for KCl depolarization.

To ensure that the uncoupling agents did not interfere directly with the luminescence effector of the photocytes, the latter were dissociated by incubating photocyte-rich autozooid tissues for 1 h in a medium containing 37.5 mg papain, 5 mg dithiothreitol (both from Sigma), 5 ml of ASW, and 5 ml of 0.37 *M* $MgCl_2$ at pH 8.0 (Holman and Anderson, 1991). After the suspensions were transferred into fresh ASW, the dissociated cells were subjected to KCl depolarization within 1–2 h of cell dissociation and their luminescent responses recorded as described above.

Dye-exchange experiments

Five sea pansies were anesthetized, their autozooid polyps were excised, and the polyp tissues were digested in the papain solution as described above. After the cell suspensions were transferred into fresh ASW, they were divided into two batches of equal volume per sea pansy, one of which was then loaded with 10 μM calcein acy-

toxymethyl ester (calcein AM) and the other with 10 μM calcein blue acetoxymethyl ester (calcein blue AM) (Molecular Probes, Eugene, OR) for 15 min at 12°C as prescribed by Leys (1995). These dyes were selected for their demonstrated ability to pass through gap junction channels (Tomasetto *et al.*, 1993) and for their propensity to become de-esterified inside cells, thus preventing their outward transmembrane diffusion. Cells were sedimented and resuspended in fresh ASW three times, then equal volumes of the two batches were mixed and the cells left to reassociate overnight at 12°C in plastic petri dishes. Just before reassociation, some batches of mixed cells were divided into two equal volumes, sedimented again, and resuspended either in fresh ASW (controls) or in ASW with 250 μM octanol. Aliquots (0.1 ml) of cell-aggregate suspensions were transferred onto microscope slides, coverslipped, and examined with a Wild-Leitz Dialux 20 fluorescence microscope. Incidence of dye exchange was assessed by scoring aggregates of three or more cells displaying at least one cell with dual calcein AM (green) and calcein blue AM (blue) fluorescence within a 1 cm^2 area.

Western blot detection of connexin43-like protein

Sea pansy colonies were anesthetized as described above and autozooid polyps, as well as rat heart tissues, were finely minced. Tissues were suspended in 15 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and homogenized with a Polytron on ice. They were centrifuged at 100 × g for 5 min to discard large debris and, after the supernatant was filtered through cheesecloth, recentrifuged at 35000 × g for 20 min at 4°C. The supernatant was discarded and the pellets were resuspended in the Tris-HCl buffer without sucrose and recentrifuged at 35,000 × g and 4°C for 15 min. This latter procedure was repeated three times. The resulting pellets were transferred to SDS reducing sample buffer (0.125 M Tris buffer with 5% SDS, 10% mercaptoethanol, 20% glycerol, and 0.005% bromophenol blue) and run in 12% SDS-polyacrylamide gels with a Bio-Rad Mini-Protean II cell according to Laemmli's (1970) method. The gels were stained with 2.5% Coomassie blue in 50% methanol and 7% acetic acid.

Immunoblots were obtained by electrophoretic transfer of gels to PUDF membranes (Bio-Rad) with 0.2-μm porosity. After exposing the blots to 3% bovine serum albumin in Tris-buffered saline (TBS: 20 mM Tris HCl with 0.5 M NaCl, pH 7.2) for 4 h to block unspecific antibody binding, they were incubated at 4°C for 24 h in a 1:1000 solution of a monoclonal anti-connexin43 (anti-Cx43) antibody (Chemicon International) in TBS. This antibody was raised against a synthetic peptide corresponding to aminoacid positions 252–270 of rat heart Cx43. That fragment is part of the COOH terminus of the protein on the cytoplasmic side, which diverges considerably among connexins (Beyer *et al.*, 1987). Blots were washed three times, 15 min per wash, in TBS and reacted with a Vectastain goat anti-mouse ABC peroxidase kit (Vector). Some blots were stained with Coomassie blue for direct comparison of protein profiles.

Effect of Cx43 antibody on luminescence

Small pieces of colonial tissue (rachis) bearing one autozooid polyp and several siphonozooid polyps (also containing photocytes) were excised from unanesthetized colonies and placed individually in assay vials containing 150 μl of ASW. To stimulate these preparations, the denuded tip of a fine, Teflon-coated silver wire was inserted in the rachis piece and another, immersed in the surrounding ASW, served as the reference electrode. The assay vial was inserted in the lightproof well of a Berthold Biolumat LB 9500T luminometer, and the opposite tips of the electrodes were connected to a Grass S9 stimulator. Spontaneous luminescent activity was first recorded as integrated light output (in millivolts) over a 30-s period. The luminescent response to a train of 10 suprathreshold monopolar pulses (25–40 V, 10 ms each, 2 Hz) was then recorded, again as integrated light output over the first 30 s after the initial stimulation pulse. The background signal of the luminometer was automatically subtracted from the measured luminescent activity.

After control responses were recorded, the ASW was removed from the assay vial and substituted with 150 μl of an ice-cold solution containing the monoclonal Cx43 antibody diluted 1:20 in ASW with 5% dimethylsulfoxide (DMSO) to allow the antibody to penetrate cells (Fraser *et al.*, 1987). Incubation was run at room temperature. Luminescent responses to electrical stimulation were recorded as above, 30–45 min after onset of incubation, then at later times specified in the Results to monitor the recovery of responses as the antibody presumably deteriorated in the tissues.

Data were analyzed by computing the ratio of stimulated over unstimulated (spontaneous) luminescent activity for each experimental condition (control and various times after exposure to antibody). This normalization was made necessary by the great variability of absolute light output between preparations. To compare ratios obtained from controls with those from antibody exposures in the same preparation, paired t tests were performed when necessary and significance level was set at $P < 0.05$.

To rule out nonspecific effects due to membrane permeabilization, unstable electrode insertion, or fatigue of preparation, we undertook sham experiments by submitting preparations to treatments as above except for

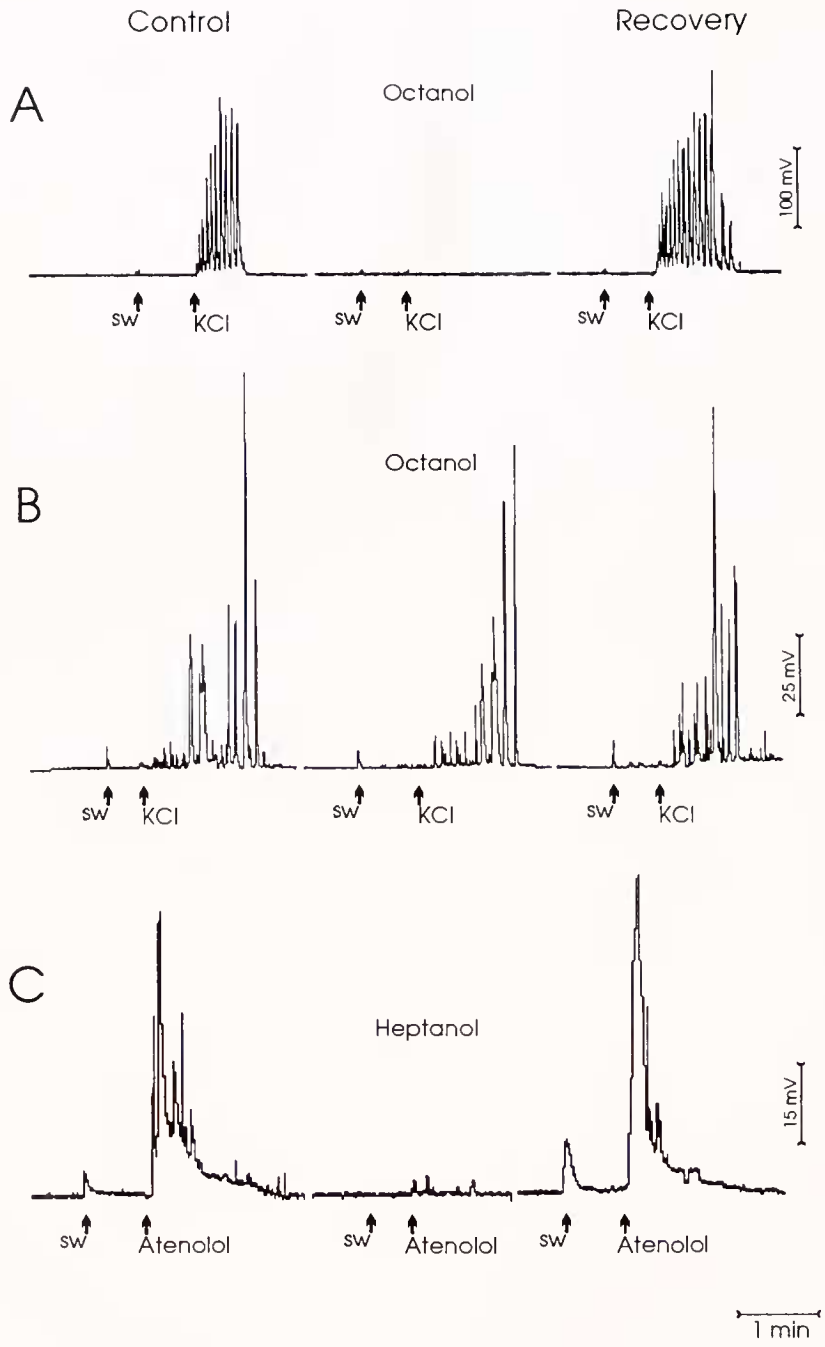

Figure 1. Photometric recordings of the effect of gap junction uncouplers on stimulated bioluminescence of *Renilla koellikeri*. (A) Effect of 0.25 m*M* octanol on *in situ* KCl-induced flash activity. (B) Effect of 0.25 m*M* octanol on KCl-induced flash activity of dissociated photocytes. (C) Effect of 0.5 m*M* heptanol on *in situ* luminescence induced by 10 μM atenolol. Spurious mechanical stimulation induced by delivery of 0.5 ml seawater (sw) was monitored throughout the experiments.

substituting fresh ASW with 5% DMSO, instead of the antibody solution, for the regular ASW of control conditions. To rule out spurious effects of macromolecules inside cells, we substituted a goat antimouse immunoglobulin (Jackson Immunochemicals), diluted 1:20 in ASW with 5% DMSO, for the Cx43 antibody in the assay vial. To verify that the effect was possible only through an intracellular route, the preparation was exposed to a Cx43 antibody solution lacking DMSO. To directly demonstrate antibody penetration, three preparations loaded

with connexin43 antibody were fixed, processed for freeze sectioning, and the sections reacted with the secondary antibody as described below.

Localization of Cx43 immunoreactivity

Autozooid polyps were excised from anesthesized colonies as above, and immersed in freshly prepared Zamboni's fixative (4% paraformaldehyde and 7.5% saturated picric acid in phosphate buffer with 2.4% NaCl, pH 7.4) for 24 h at 4°C. After three rinses in PBS, followed by immersions in PBS with 15% and 30% sucrose, the polyps were embedded in O.C.T. compound (Miles) and frozen by immersion in dry-ice-chilled isopentane. Sections 15 μm thick were made with a Hacker-Bright cryotome. The sections were rinsed in three 15-min changes of PBS, followed by pretreatment in PBS with 0.2% Triton-X-100 and 1% bovine serum albumin (BSA). Incubation was carried out for 24 h at 4°C in the Cx43 monoclonal antibody diluted 1:200 or 1:100 in PBS with Triton-X-100 and BSA as above. After rinsing in PBS as above, sections were incubated in PBS for 1 h at room temperature in Cy3-conjugated goat anti-mouse immunoglobulin diluted 1:100. Sections were then rinsed in PBS as above and mounted in a 1:3 mixture of glycerol-PBS before being examined with a Wild-Leitz Dialux 20 fluorescence microscope equipped with a rhodamine filter. Control sections were processed as above except for omitting the primary antibody from the incubation solution.

Electron microscopy

Autozooid polyps were excised from anesthesized colonies as described above. They were immersed in a freshly prepared fixative solution containing 2% glutaraldehyde and 4% paraformaldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.4) with 2.4% NaCl for 1 h at room temperature. After rinsing tissues in buffer, they were postfixed in 1% OsO_4 in cacodylate buffer for 1 h. Tissues were dehydrated in graded ethanol and propylene oxide, and embedded in Epon 812. Sections were cut with a diamond knife on an LKB ultramicrotome and deposited on 100- and 200-mesh stainless steel grids (JBEM Inc.). The sections were counterstained with uranyl acetate and lead citrate and examined with a JEOL-100S electron microscope.

Results

Effect of gap junction uncouplers on evoked luminescence

The light emission evoked in sea pansy autozooids by KCl depolarization appeared as a series of flashes of varying amplitude and frequency (Fig. 1A,B). These responses were either considerably reduced or abolished in a reversible manner in the presence of octanol, heptanol, or sodium acetate-seawater (Figs. 1A, 2). Photocyte-enriched dissociated cell preparations responded to KCl depolarization with series of irregular flashes (see also Germain and Anctil, 1988). In contrast to responses of photocytes *in situ*, the KCl response of dissociated photocytes was unaffected in the presence of octanol or heptanol (Figs. 1B, 2). Field electrical stimulation induced facilitated flashes on a one-to-one basis with stimulation pulses, and this type of response was also largely and reversibly abolished in the presence of any of the three uncouplers (not shown). Stimulation of the β2-like adrenergic receptors of the sea pansy is known to induce bioluminescence (Awad and Anctil, 1993). Exposure of tissues to isoproterenol, a nonselective β-adrenergic agonist, or to atenolol, a selective β2-adrenergic agonist in the sea pansy (Awad and Anctil, 1993), induced luminescence in the form of glows and superimposed flashes, a response that was greatly and reversibly reduced in the presence of heptanol (Fig. 1C).

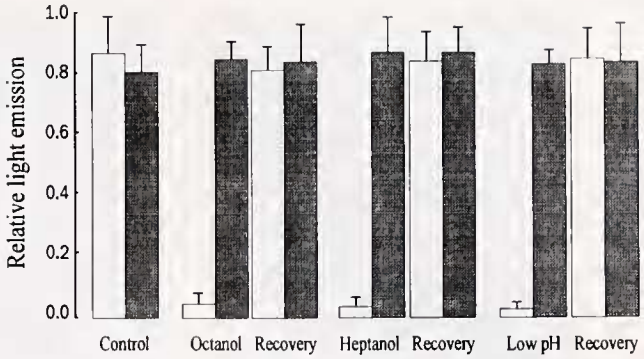

Figure 2. Histogram summarizing data on the effect of exposure to gap junction uncouplers on KCl-induced bioluminescence of intact tissues (open bars) and dissociated photocytes (shaded bars) of *Renilla koellikeri*. Data are expressed as means ± SE (n = 6 experiments) of light output ratio relative to controls.

Dye exchange

Cells loaded with calcein AM or calcein blue AM were strongly fluorescent, and their fluorescence did not fade significantly over a period of 24 h. When cells loaded with either of the two dyes were allowed to reaggregate for 15 h, many of them exhibited both the green fluorescence of calcein AM and the blue fluorescence of calcein blue AM (Fig. 3). Among those were identifiable cells such as endodermal granular cells known to harbor luminescence-associated β2-like adrenergic receptors (Awad and Anctil, 1994) and nerve cells (Fig. 3). Of 1465 cell clusters (three cells or more) scored in 10 aliquots of mixed cell suspensions from three sea pansy colonies,

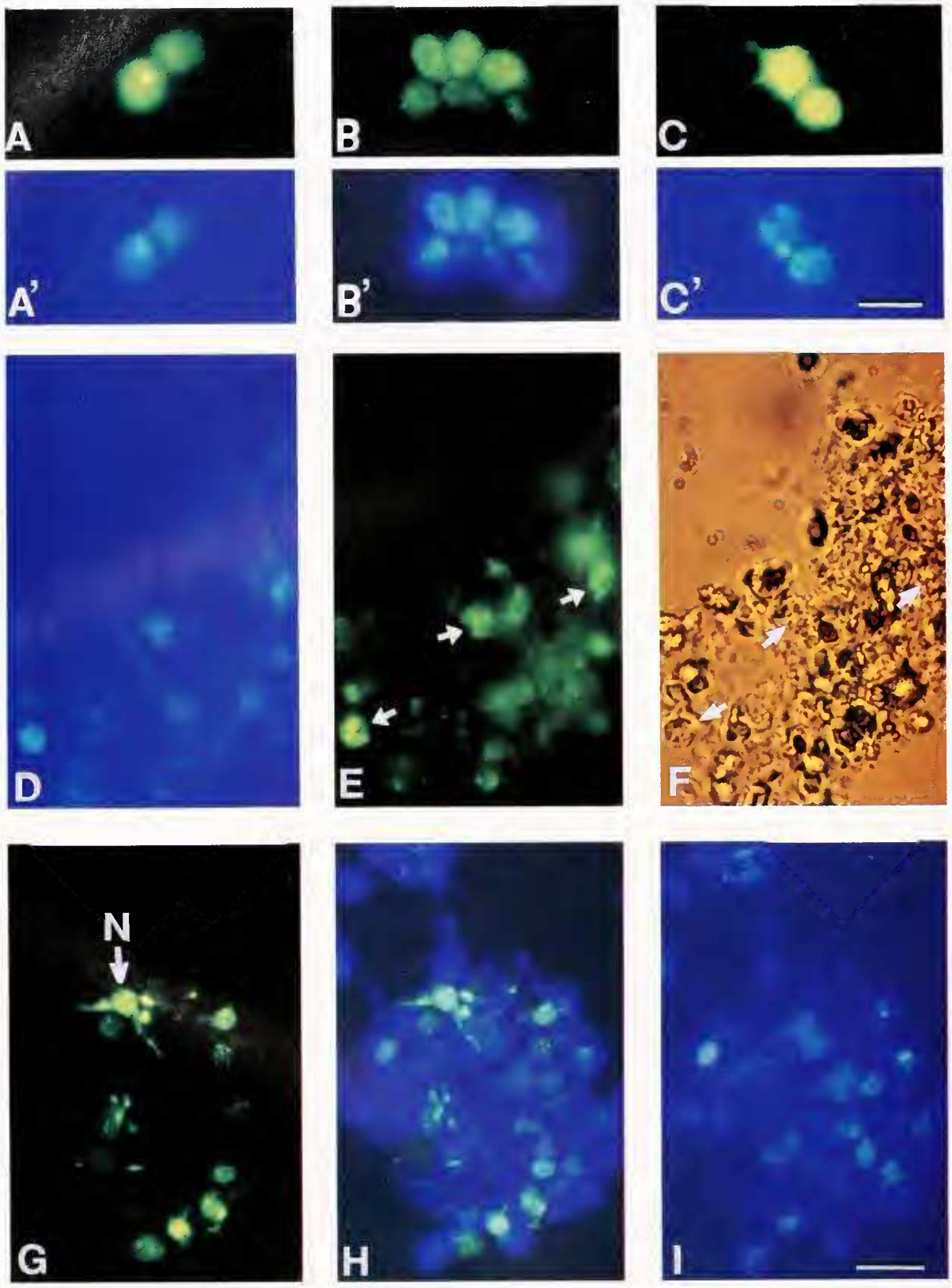

Figure 3. Demonstration of dye transfer between reaggregated sea pansy cells. Dissociated autozooid polyp cells were loaded separately with calcein AM and calcein blue AM and plated together to allow reaggregation and dye passage between cells within a reaggregate. Upper panel shows dye exchange between

two-thirds [66.5% ± 3.9% (X ± SEM, n = 10 aliquots)] included at least one cell in which the dyes were co-localized. In contrast, mixed cell suspensions allowed to reaggregate in the presence of 250 μM octanol for 15 h showed zero incidence of dye exchange. In addition, the incidence of cell clusters was reduced to one-third (30.7% ± 4.9%, n = 5 aliquots) of that in untreated suspensions from the same cell batches. A partial reversal of the octanol effect was recorded when reaggregation was prolonged to 48 h, with low incidence of dye exchange (13.9% ± 2.2%, n = 5) and an increase in the treated/untreated ratio of cell aggregates (44.2% ± 5.8%, n = 5).

Detection of a connexin-like protein

Because the results from experiments with uncouplers and dye exchange suggested the involvement of gap-junction-like communication in the sea pansy, we next looked for gap junction proteins. We chose to focus on Cx43 because preliminary Northern blot analyses in another anthozoan, the sea anemone *Haliplanella*, had revealed hybridization products with rat heart Cx43 but not with rat liver Cx32 cDNA (Hessinger *et al.*, 1992).

As shown in Figure 4 (lanes 1 and 2), Western blots of membrane protein extracts from the sea pansy revealed two antigen bands that bound the Cx43 antibody, one with an apparent molecular weight of ~43 kDa and another with twice that molecular weight. Both bands co-electroeluted with the rat heart 43 kD connexin and its presumptive dimer (Fig. 4, lane 3). The rat heart bands had a density more-or-less similar to those of the sea pansy.

Effect of Cx43 antibody on luminescence

The antibody was successfully and consistently introduced into cells of the sea pansy by permeabilization with DMSO; this result was demonstrated by visualization of a diffuse tissue fluorescence in loaded preparations that were fixed after experiments and reacted with Cy3-conjugated goat anti-mouse antibody. Under our loading conditions, the tissues responded normally to electrical stimulation with facilitating flashes as reported above. Whether in the presence or absence of 5% DMSO, the unloaded preparations also exhibited a facilitation of the integrated luminescent response to a second challenge 30–45 min after the first (Fig. 5).

The luminescent responses of all preparations loaded for 30 min with the Cx43 antibody were nearly abolished for at least 90 min (Fig. 5). There was a partial but significant recovery 5–7 h after loading, presumably due to antibody degradation, and another response decline more than 10 h later, probably resulting from tissue deterioration (Fig. 5) brought about by conducting the experiments at room temperature. Loading preparations with a goat anti-mouse immunoglobulin instead of the Cx43 antibody or exposing preparations to the Cx43 antibody without DMSO did not significantly alter the luminescent response. Moreover, exposing tissues to the antibody solution did not, by itself, elicit any luminescence.

Immunohistochemical localization of Cx43-like antigen

Because it seemed that the Cx43 antibody specifically recognized an antigen behaving like rat heart Cx43 in Western blots and acted like a gap junction uncoupler to block luminescent responses, we undertook to determine by immunohistochemistry whether Cx43-like immunoreactivity is present in the photocyte-rich endoderm.

Specific Cx43 immunoreactivity was widespread but more abundant in the endoderm (Fig. 6A–C) and in the mesogleal nerve-net (Fig. 6D) than in the ectoderm. Immunofluorescence was lost in sections unexposed to the primary antibody; the only exceptions were some ectodermal gland cells that exhibited nonspecific reactivity. In both autozooid and siphonozooid polyps, photocytes are clustered in specific locations of the endodermal layer where cells filled with highly refringent granules (presumably acting as diffuse reflectors for the light emission) predominate (Anctil *et al.*, 1984; Awad and Anctil, 1994). In autozooids these locations are at the base of each of the eight tentacles and at the base of the corresponding eight septa of the autozooid column (Morin, 1974). It was in these parts of the endoderm that the density and intensity of immunoreactive sites appeared to be the greatest (Fig. 6A,B).

The other site of abundant immunoreactivity was the

all cells of three small clusters, with view of calcein AM dye (A–C) and calcein blue AM dye (A'–C'). Cells in C,C' are granular endodermal cells. Middle panel shows a large cluster of cells in which only a few cells exchanged dye, such as the three cells filled with calcein blue AM (D) that are indicated by arrows in views of their calcein AM fill (E) and of the cell mass in bright field (F). Note in F the presence of symbiotic brown unicellular algae. Lower panel shows a cell cluster from a preparation in which cells were allowed to reaggregate in the presence of 250 μM octanol. G and I are views of calcein AM and calcein blue AM dyes, respectively, and H is a double exposure of both dyes. N is a neuron with cilium and bifurcating axon. Note that no dye exchange occurred. Bar in upper panel = 15 μm, and bar in lower panel = 20 μm (D–I).

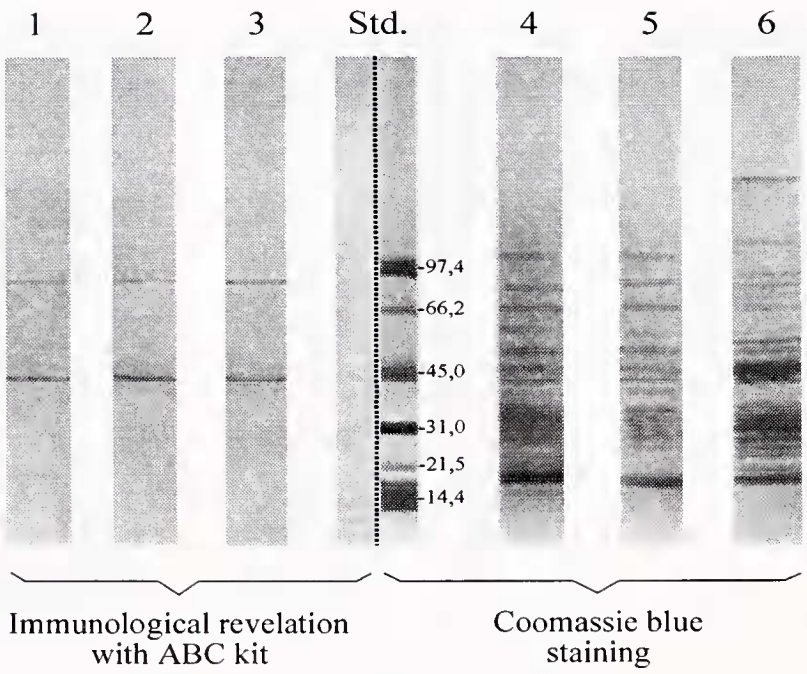

Figure 4. Immunoblot analysis of connexin43-like protein in sea pansy membrane homogenates. Lanes 1 and 2 show binding of monoclonal Cx43 antibody to the 43 kDa monomer and the 86 kDa dimer from homogenates of two sea pansies, and lane 3 shows the same from a rat heart homogenate. Lanes 4–6 are Coomassie blue stained blots from the homogenates of lanes 1–3, presented in the same order.

mesogleal nerve net, a meshwork of neurons and amoebocytes embedded in the collagen-based jelly, the mesoglea, that separates ectoderm from endoderm (Fig. 6D). Unlike the luminescent endoderm, in the mesoglea immunoreactivity was inconsistent, being strong in some locations (Fig. 6D), weak or absent in others. The immunoreactivity appeared as tiny fluorescent dots distributed in stringlike fashion along tracts of amoebocytes and neurites. The size of the fluorescent dots was in the submicrometer range. Lack of resolution made it impossible to determine if the immunoreactivity was associated with either amoebocytes or nerve cells, or both.

The pattern of immunoreactivity in the luminescent endoderm was polymorphic. Reactivity usually appeared as punctate immunofluorescence (Fig. 6A), as expected of minute structures the size of gap junctions and as observed in tissues examined in this way, including those of the cnidarian *Hydra* (Fraser *et al.*, 1987). The fluorescent dots varied in diameter but, as in the mesoglea, all were in the submicrometer range. Owing to the thickness of the sections and the multifocal planes where these small cells are located, the distribution of the reactive dots appeared generally random. Occasionally, however, cells containing refringent granules appeared to be associated with punctate immunoreactivity at their boundaries (Fig. 6A). Less frequently, immunoreactive endodermal cells were individually recognizable, perhaps because contiguous cells lacked reactivity and allowed an unencumbered view. In this case the immunofluorescence was associated with cell boundaries, cell contour fluorescence appearing as a fine threadlike assembly (Fig. 6C).

Ultrastructural observations

The photocyte-rich endoderm was examined by electron microscopy for evidence of specialized contacts between cells. An exhaustive search failed to uncover any structure corresponding to descriptions of conventional gap junctions such as those described in hydrozoans (Hand and Gobel, 1972; King and Spencer, 1979). However, in addition to septate junctions (Fig. 7A), small zones of close membrane apposition were frequently seen between endodermal cells (Fig. 7A–C). These contact zones were observed between a variety of endodermal cells, including granular cells (not shown), myoepithelial cells (Fig. 7A), photocytes (Fig. 7B,C) and digestive cells (Fig. 7C). These generally had a contact area of 20–50 nm and an intermembrane gap of 2–4 nm, but lacked any other specialization except for densities in the gap zone. Less frequently, larger contact zones, up to 100 nm in length with intercellular gap spaces of 5–6 nm, were characterized by gap densities of regular periodicity (Fig. 7B).

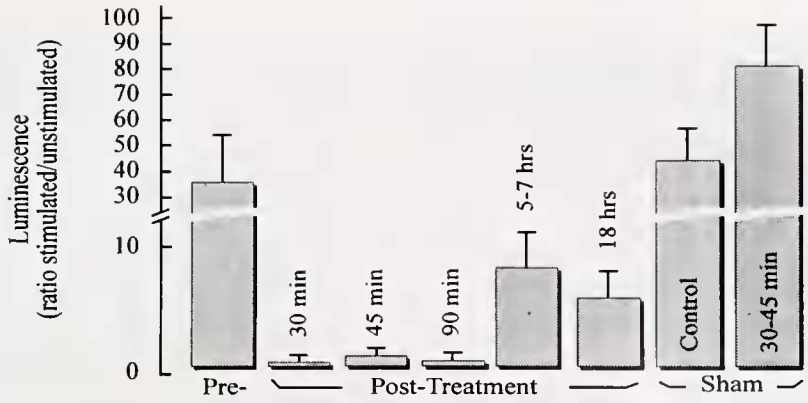

Figure 5. Effect of Cx43 antibody treatment on bioluminescence elicited in sea pansy tissue preparations by electrical stimulation. Pretreatment represents response to electrical stimulation in normal ASW immediately before onset of antibody penetration experiments. Sham represents experiments in which pretreatment was as before (control) but treatment consisted of exposing the preparations to ASW + 5% DMSO. Data from responses 30 and 45 min after exposure were pooled. Bars represent means ± SEM (n = 6, except at 5–7 h and 18 h when n = 4 and 3, respectively).

Discussion

The results of this study provide converging evidence for the existence of a connexin-like gap junction protein in the sea pansy and for the involvement of connexon-mediated intercellular coupling in the transmission of luminescence effector signals. This and other evidence (Hessinger *et al.*, 1992), while confirming the failure to observe typical gap junction plaques in anthozoans, challenges the notion that anthozoan cells "have little if any capability for direct electrical or metabolic communication" (Mackie *et al.*, 1984).

Intercellular coupling occurs and is necessary for mediation of luminescence signaling

The conventional demonstration of dye coupling after intracellular injection of Lucifer yellow was not feasible because of the technically challenging small size and instability of penetrated sea pansy cells. Our prior experience with suspensions of dissociated sea pansy cells indicated that reassociation into clusters of varying sizes occurred. We took advantage of this property in experiments designed to allow exchange of two vital dyes (calceins) between reassociated cells. The observation that a substantial number of cell clusters included cells doubly labeled with calcein AM and calcein blue AM under these conditions indicates that dye exchange occurred and, consequently, that some form of coupling is effected between these cells. The possibility that dye colocalization results from dye leaking out of cells and into cells loaded with the other dye is highly unlikely. The calcein dyes are noteworthy for their effective retention inside viable cells and no evidence of leaching, such as reduction of the fluorescent signal, was observed in the course of these experiments. The suppression of dye exchange in the presence of the uncoupler octanol also reinforces the notion that dye exchange occurred via connexon-related channels. It should be emphasized, however, that these results provide strong evidence that coupling is feasible between reassociated sea pansy cells; whether this coupling in fact occurs in intact tissue remains to be resolved.

Luminescent responses of intact autozooids to KCl depolarization, β-adrenergic activation, and electrical stimulation were all inhibited in a reversible manner by octanol, heptanol, and low pH (6.5). These agents are known for their blocking effect on coupling *via* gap junctions (Johnston *et al.*, 1980; Turin and Warner, 1980; Spray *et al.*, 1982) and have perturbing effects on the ultrastructure of connexon arrangements (Délèze and Hervé, 1983). They not only inhibited the KCl-induced luminescent response of endodermal cell clusters of *Obelia*, but also blocked the intercellular spread of injected Lucifer yellow in these preparations (Dunlap *et al.*, 1987). The possibility exists that the observed effects are due to interference with participants in the luminescent system other than intercellular coupling. This is unlikely, however, because the luminescent responses of dissociated photocytes were unaffected by the uncoupling agents. If KCl depolarized photocytes directly in intact tissues, uncoupling agents would not be expected to affect luminescence. The effectiveness of uncoupling agents on *in situ* luminescence suggests that KCl triggers luminescence only *via* nerve-net depolarization as occurs following electrical stimulation. If KCl directly depolarized photocytes, a sustained glow response would

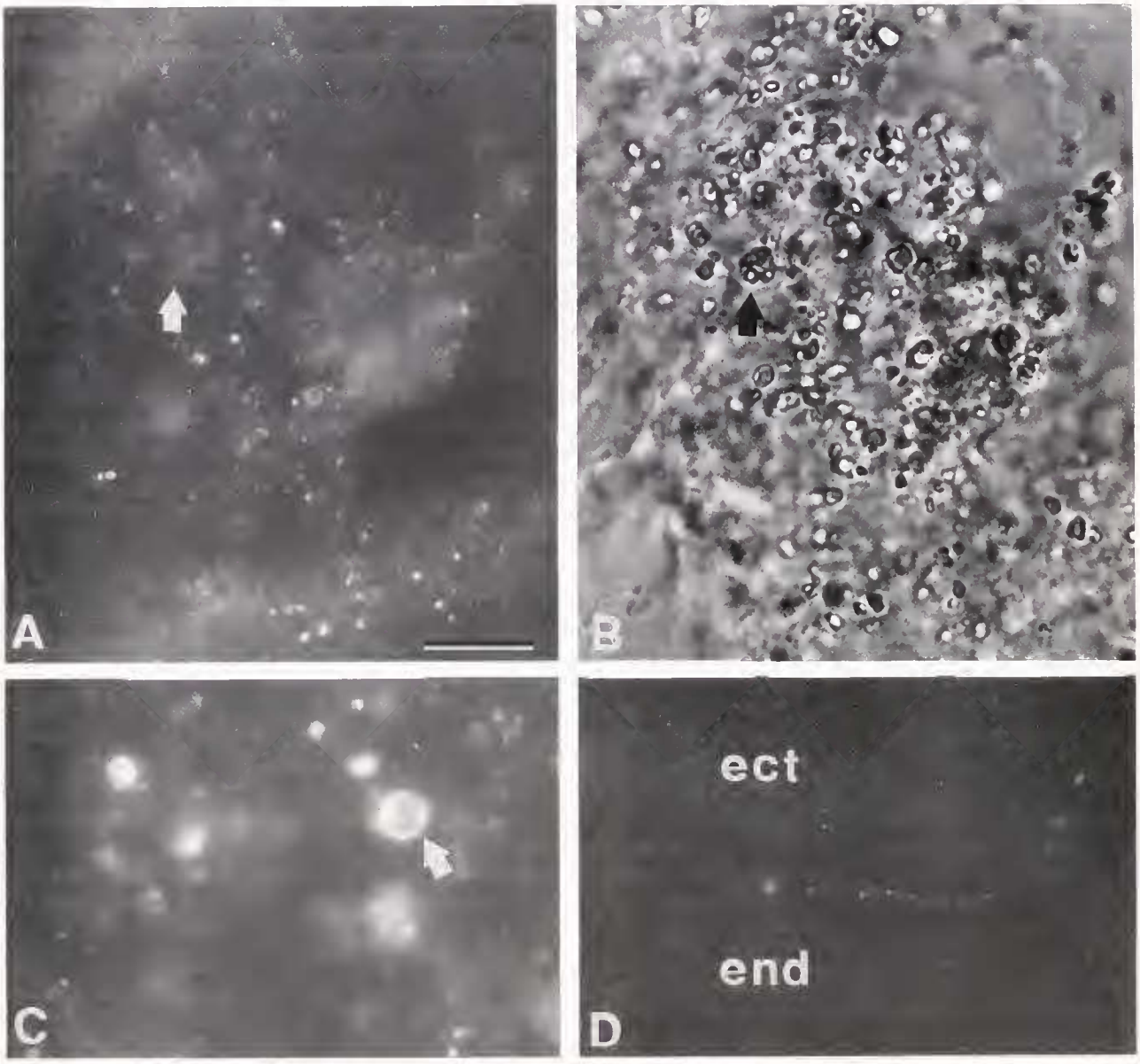

Figure 6. Connexin43 immunofluorescence in the luminescent endoderm (A–C) and mesoglea (D) of autozooid polyps of the sea pansy. (A) Punctate connexin43 immunofluorescence in luminescent endoderm. (B) Phase contrast view of field in A, showing details of refringent granule distribution and morphology. Note that granular cell by arrow in B lacks immunofluorescence in A (arrow) except for apparent punctate reactivity along its contour. (C) Endodermal cells with immunofluorescent contour, one of which (arrow) is in sharp focus and exhibits a string of tiny dots. (D) String of punctate immunofluorescence in mesogleal amoebocyte tract (in which lies the nerve net), sandwiched between non-immunoreactive ectodermal (ect) and endodermal (end) layers. Calibration bar = 10 μm (A–C), 9 μm (D).

be expected; instead, KCl produced phasic luminescent responses. Thus the necessity of the integral luminescent tissue for the blocking effect of the uncouplers and the consistency of action of all three uncouplers on luminescent responses obtained by different means can best be explained by the involvement of some form of intercellular coupling in mediating the luminescent signal.

A connexin43-like protein is present in sea pansy tissues

If intercellular coupling sensitive to gap junction blockers is involved in sea pansy luminescence signaling, then one should be able to detect a protein capable of assembling into connexons for this coupling. There is *a priori* no reason to assume that such a protein should

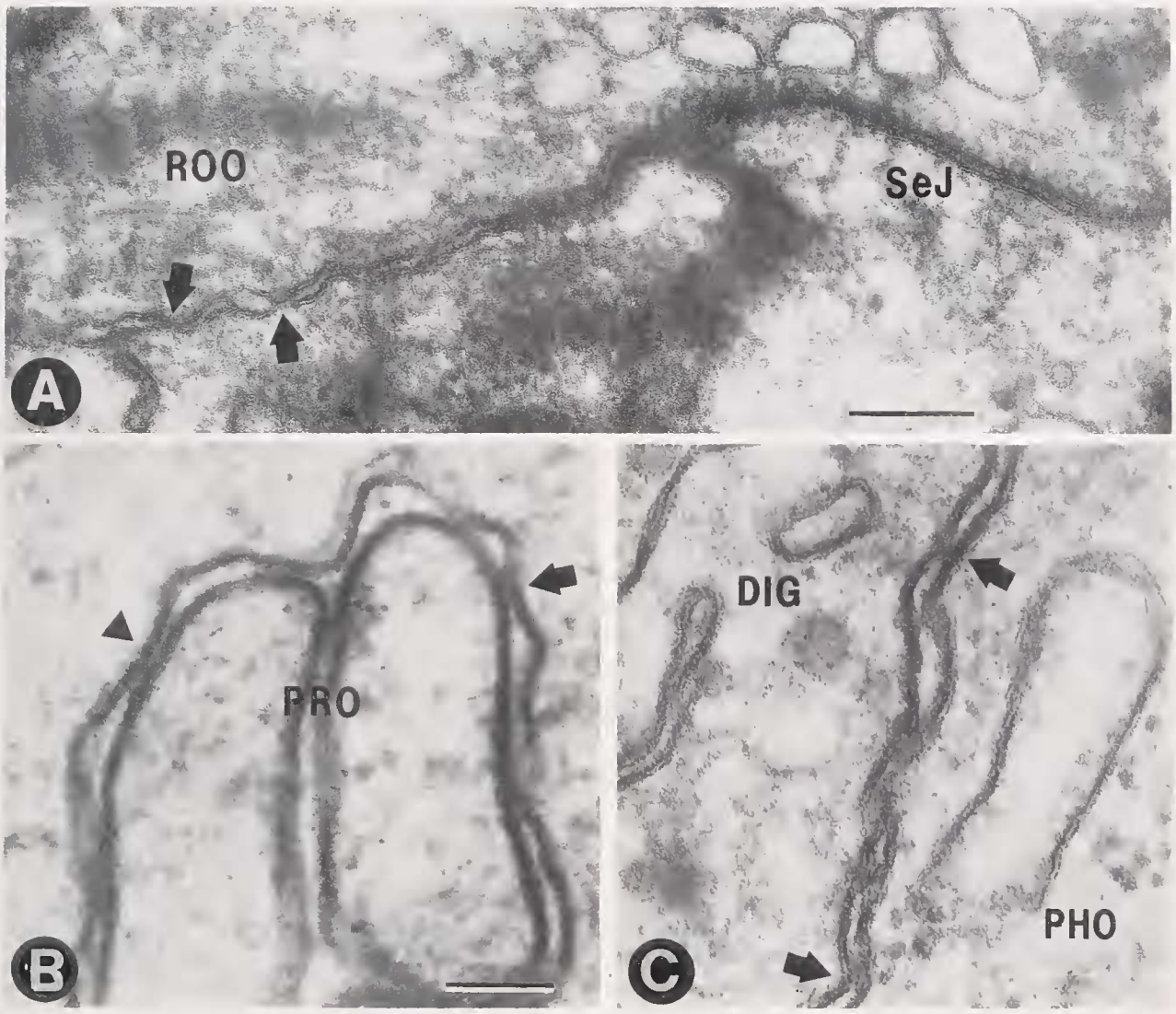

Figure 7. Electron micrographs showing small zones of close apposition (arrows) between plasma membranes of contiguous endodermal cells. (A) Between two myoepithelial cells also connected by a septate junction (SeJ); one of the cells contains a motor ciliary rootlet (ROO). (B) Between a photocyte and two processes (PRO) of unidentified endodermal cells. Note also contact zone with intercellular gap densities displaying periodicity (arrowhead). (C) Between a digestive cell (DIG) and a photocyte (PHO). Calibration bars = 0.2 μm (A), 0.1 μm (B,C).

belong to the vertebrate connexin family, and a case has been made for the existence of a distinct family of invertebrate gap junction proteins based on sequence homologies of *Drosophila* and *Caenorhabditis* gene products suspected of involvement in electrical synapses (Barnes, 1994). More direct evidence was recently adduced for the involvement in intercellular coupling of two such proteins derived from *Caenorhabditis* cDNA sequences and exhibiting a membrane topology strikingly similar to that of connexins (Starich *et al.*, 1996). However, the detection in *Hydra* of a protein recognized by antibodies to rat-liver gap junction proteins (Fraser *et al.*, 1987) and in sea anemones of a mRNA hybridization signal probed with rat heart connexin cDNA (Hessinger *et al.*, 1992) lends support to the notion that the connexin family may extend phylogenetically to the phylum Cnidaria.

The Western blot detection in sea pansy tissues of a protein recognized by a monoclonal antibody to a peptide segment of rat heart Cx43 provides further evidence that a Cx43-like protein similar to that of mammals exists in anthozoans. The detection was highly specific: no other band appeared on the immunoblot except for the dimeric form of the protein, also seen in simultaneously processed blots from rat heart extracts. The peptide seg-

ment of connexin43 that served to raise the antibody is part of the cytoplasmic carboxyl terminus that presents no homology with rat liver Cx32 (Beyer *et al.*, 1987) and therefore constitutes a "signature" of that particular connexin. In contrast, that same fragment appears to have been conserved among vertebrate connexin43s. This conservation is demonstrated by the 63% identity of aligned amino acids between the corresponding sequences of the amphibian *Xenopus* and rat heart Cx43s (Gimlich *et al.*, 1990). Our results suggest that this structural conservation extends to lower invertebrates. To our knowledge, no connexin-like protein has yet been detected in invertebrates other than cnidarians; more work is thus needed to substantiate this hypothesis.

A Cx43-like protein is necessary for mediation of luminescence signaling

Use of specific antibodies raised against gap junction proteins to derive the functional role of gap junctions was pioneered by Warner *et al.* (1984) in a study of the role of gap junctions in embryogenesis. The method also proved useful in establishing a relationship between gap junction activity and developmental processes in the cnidarian *Hydra* (Fraser *et al.*, 1987). We took advantage of the detection of Cx43-like immunoreactivity in the sea pansy to use the Cx43 antibody in the same fashion and look for evidence of disruption of luminescent activity. Thus we found that the successful introduction of the antibody into sea pansy cells by permeabilization reversibly inhibited luminescent responses. The effect was not due to the permeabilization agent (DMSO) or to the antibody acting on the external surface of cells because we demonstrated that DMSO alone or the antibody delivered without DMSO did not affect luminescent responses. The possibility that the blocking effect was due to an unspecific inhibition of cellular activity by introducing macromolecules in cells was discarded when substitution of another antibody for the anti-connexin failed to block luminescent responses. Thus the Cx43 antibody effect appears to be specific and to occur from inside the cells, as predicted by the fact that the antibody recognizes a fragment of the connexin located on the cytoplasmic side (Beyer *et al.*, 1987). Although we are unaware of any previous evidence that this antibody blocks gap junction channels formed of Cx43, an antibody raised against a cytoplasmic Cx43 fragment composed of amino acids 313–324 was an effective blocker of such channels (Becker *et al.*, 1995).

The convergent results from the experiments with gap junction uncouplers and with the Cx43 antibody suggest that a Cx43-like protein is involved in mediating luminescence propagation through cell-to-cell coupling in the sea pansy. The acute inhibitory effect of the antibody on electrically stimulated luminescence means that the antibody binds to an antigen widely distributed in the luminescent endoderm or in the nerve net that propagates the excitatory signals responsible for lighting up photocytes along the signal path (Anderson and Case, 1975).

The Cx43-like protein is localized in the luminescent endoderm and in the mesogleal nerve net

Specific Cx43-like immunoreactivity was present in all tissue layers of autozooid polyps but was more prevalent in the luminescent endoderm and in the mesogleal nerve net. Reactive sites had a predominantly punctate appearance and, in the mesogleal nerve net, a linear dotlike distribution compatible with the labeling of connexon patches (Fraser *et al.*, 1987). The higher density of reactive sites in the luminescent endoderm is not due to the presence of numerous refringent granules in cells associated with the photocytes. These granules were not immunofluorescent, and their size (1–4 μm) exceeds that of the punctate reactive sites. Thus the preferential distribution of Cx43-like immunoreactivity in this location concurs with the potent inhibitory effect of the Cx43 antibody on luminescence and suggests that a protein of the Cx43 family is an integral part of signaling among those endodermal cells. It was not possible to determine whether all endodermal cells of the luminescent zone were immunoreactive and, consequently, all potentially coupled. What is clear is that cells with refringent granules are prominent among the reactive ones. Given that the latter appear to express the β2-like adrenoceptors associated with luminescence control (Awad and Anctil, 1993, 1994), the likelihood is great that intercellular coupling involving Cx43-related connexons participates in downstream transmission of signals to the photocytes themselves. This arrangement would not be unique to the sea pansy—β-adrenoceptors acting *via* cyclic AMP have been implicated in the modulation of gap junction recruitment in mammalian cell lines (Radu *et al.*, 1982).

The presence of Cx43-like immunoreactivity in the mesogleal nerve net is difficult to interpret because labeling had a patchy distribution and the cellular source of labeling is unclear. Although the presence of a connexin-like protein does not necessarily confer intercellular coupling attributes, such a functional implication remains a possibility. If the amoebocytes contained a Cx43-like antigen, it would be possible to envisage a metabolic and electrical coupling function equivalent to that reported in glial cells of the mammalian CNS (Dermietzel *et al.*, 1991). If labeling is at least in part associated with neurons, the possibility of electrical coupling within the nerve net seems irreconcilable with the high magnesium sensitivity of nerve-net conduction, and interneuronal transmission *via* chemical synapses seems more likely

in anthozoans (Anderson and Case, 1975; Satterlie and Spencer, 1987). Through-conduction of mesogleal nerve-net pulses seems to imply that no interneuronal facilitation is necessary in the sea pansy (Anderson and Case, 1975). Thus coupling between cells of the mesogleal nerve net (amoebocytes and neurons) *via* connexon channels may contribute to a through-conduction feature that is otherwise difficult to account for solely on the basis of chemical neurotransmission.

Possible ultrastructural substrates of junctions involving connexons

An electron microscopic survey of polyp tissues in the sea pansy has failed to unveil any structure resembling conventional gap junctions. However, as admitted by Mackie *et al.* (1984), "the failure to observe gap junctions in these groups could merely mean that the junctions are very small, consisting of isolated connexons, or small groups of them." It was already noted above that the punctate Cx43-like immunofluorescence in the endoderm of the sea pansy was in the submicrometer range, thus substantially smaller than corresponding signals associated with true gap junction plaques (1–5 μm; see Fraser *et al.*, 1987; Dermietzel *et al.*, 1991; Risek *et al.*, 1994). Therefore, the many small apposition zones observed at adjoining endodermal cells are possible substrates for the Cx43-like immunofluorescence. Such point contacts of limited length (50 nm or less) with a very narrow gap (2–4 nm) often containing densities may be analogous to miniature gap junctions. It is interesting that ctenophore apical zonular junctions, which are structurally similar to the sea pansy's close apposition zones, were described as minigap junctions (Hernandez-Nicaise, 1991). Thus in cnidarians, as in other metazoans, identifiable gap junctions may not be the only venue of cell-to-cell coupling, and the point contacts of close apposition described here may serve as a functional alternative using a similar constitutive membrane protein.

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